The Effect of β-Glucans on Cell Association and Intracellular Survival of *Mycobacterium bovis* BCG in Human Macrophages

A Senior Honors Thesis

Presented in Partial Fulfillment of the Requirement for Graduation *with Distinction* in Biology in the Undergraduate College of Biological Sciences of The Ohio State University

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The Ohio State University
June 2007

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Background and Abstract:

In 2004 there were an estimated 1.7 million deaths from tuberculosis (TB). Due to improper use of antibiotics, many drug resistant forms of *Mycobacterium tuberculosis* (*M.tb*), the causative agent of TB, have risen around the world, making this disease a leading cause of death among infectious disease agents. *M.tb* remains dormant in healthy hosts and becomes active when the immune system is weakened. The bacterium is an intracellular pathogen of macrophages. *M.tb* uses complement receptor 3 (CR3), among others, for complement-opsonized and non-opsonized phagocytosis by human macrophages and evades host defenses by replicating intracellularly in the phagosome. The polysaccharide β-glucan has been found to competitively inhibit binding to CR3 by incoming pathogens and allows for their clearance through other mechanisms. For example, it has been shown to up-regulate the production of pro-inflammatory cytokines and promote both anti-microbial and anti-tumor activities, most likely through its binding to the β-glucan receptor, Dectin-1, expressed on host macrophages. Because of its effects on CR3 and the innate immune response, we hypothesize that β-glucans would enhance activity against *M.tb* by inhibiting CR3-mediated phagocytosis and up-regulating human macrophage microbicidal activities. The effects of β-glucans were tested on human monocyte-derived macrophages (MDMs) which were obtained by allowing differentiation of blood monocytes isolated from healthy donors. The MDMs in monolayer culture were infected with the attenuated *Mycobacterium bovis* BCG (vaccine strain), a highly genetically-related model bacterium to *M.tb*, in the presence or absence of different β-glucan preparations [PGG (soluble) and WGP (insoluble)] supplied from Biothera (Eagan, Minnesota). After a 2 h infection, the infected cell monolayers were

fixed and mycobacteria were stained with auramine-rhodamine dye to assess bacterial association (bound and internalized) with macrophages by fluorescence microscopy. Alternatively, following infection, the MDMs were lysed at different time points (24 h, 48 h, 72 h), the lysates spread on agar plates for bacterial growth, and colony forming units (CFUs) determined to assess bacterial intracellular survival. This *in vitro* study produced preliminary results showing an antimicrobial effect by β-glucans against *M. bovis* BCG in MDMs as demonstrated by both bacterial survival and cell association assays. This preliminary study shows that β-glucans may possess therapeutic potential to treat TB infection.

Introduction:

Tuberculosis as a Disease: Tuberculosis (TB) is a contagious disease spread from infected individuals through aerosol droplets which are inhaled by healthy humans in close proximity and deposited into the lungs. One third of the world's population is infected with the bacterium (bacillus) which causes TB and nearly 2 million people worldwide die annually from this disease (1). In the majority of infected individuals, the bacillus will remain dormant for years without noticeable symptoms (latent TB), and reactivate when the host's immune system is compromised (reactivation TB) (Figure 1). TB is the second most deadly infectious disease in the world (23,25). When active, the TB bacillus forms a particularly lethal combination with human immunodeficiency virus (HIV) in AIDS patients due to the host's weakened immune system. The high rate of mortality of HIV-infected individuals in African countries correlates directly with an

increase in TB-afflicted individuals in this region since 1990 (1). In 1993, the rise of TB was so severe that the World Health Organization (WHO) declared the disease a global health emergency. Due to improper use of antibiotics, drug resistant TB has arisen worldwide. Bacterial strains which cause TB, showing resistance to at least one of the two most commonly used TB drugs, isoniazid and rifampin, have been found in every country that has been surveyed by WHO. Multi-drug resistant TB (MDR-TB), unaffected by both isoniazid and rifampin, has arisen worldwide making up 20% of known cases (2). In 2005, extensively drug resistant TB (XDR-TB) was first reported as having resistance to isoniazid and rifampin, to fluoroquinolones, and to at least one of the following second line injectable drugs: amikacin, capreomycin, or kanamycin. XDR-TB makes up about 3% of TB cases worldwide and has increased in foreign-born individuals in the U.S. since 1999 (3). The gradual rise in MDR-TB and the recent emergence of XDR-TB add to the continued difficulty in controlling the spread of this disease. The increasing rate of developing resistant strains makes the need for new therapies essential in combating TB worldwide.

Mycobacterium tuberculosis as a Causative Organism: TB is caused by the slow-growing, acid-fast bacillus *Mycobacterium tuberculosis* (*M.tb)*. *M.tb* is an obligate aerobic bacterium and an intracellular pathogen of macrophages. It enters the human body through inhalation and deposits into the oxygen-rich alveolar sacs of the lungs (25). One of the first groups of cells that bacteria come in contact within the lungs is alveolar macrophages. These phagocytic cells have unique attributes which make them efficient in combating incoming particles in the airways with controlled immune responses (5).

Phagocytosis of the bacteria, maturation of the bacterial phagosome, and generation of pro-inflammatory responses are critical aspects in controlling *M.tb* infection (25). Microbes are recognized by pattern recognition receptors (PRRs) on cells of the immune system, including neutrophils, dendritic cells, natural killer cells and macrophages. These PRRs detect structural components on pathogens and stimulate an immune response to inhibit infection. Among the PRRs important for the recognition of *M.tb* by alveolar macrophages are the mannose receptor (MR), Complement Receptor 3 (CR3) and Toll-like receptors [TLRs] (5,36). Both the MR and CR3 have been found to play a critical role in the phagocytosis of *M.tb* by human macrophages (17,26). The ligand for the MR is mannose-capped surface unit lipoarabinomannan, (ManLAM) found on *M.tb* and *Mycobacterium bovis* BCG (*M. bovis* BCG) (17,27). *M.tb* is phagocytosed by CR3 in both a C3bi opsonic and non-opsonic-dependent manner (6). Once phagocytosed by alveolar macrophages, most bacteria are generally degraded by acidic hydrolases upon fusion with the lysosomal compartment within the macrophage (phagolysosome fusion). Macrophages also degrade engulfed bacteria through microbicidal functions mediated by cytokines. These functions include an oxidative burst through generation of reactive oxygen intermediates (ROI) along with reactive nitrogen intermediates [RNI] (25). Non-phagocytic TLRs activate inflammatory cytokines and development of antigen-specific immunity upon ligand binding with *M.tb*. Notably, TLR2 (dependent on myeloid differentiation factor 88 or MyD88) has been found to work in concert with other receptors to produce tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6), cytokines important in initiating a host inflammatory response (15). TNF-α is a macrophage activating factor which generates nitric oxide, a critical component in

destroying pathogens. Recent studies have shown that TLR2-deficient mice have impaired immune responses to mycobacterial species (15), along with decreased TNF-α secretion (36). The outcome of mycobacterial infection within the lungs has been identified to be dependent on TLR2 activation (10).

M.tb's pathogenicity comes from its ability to evade immune responses. This organism has evolved into an extremely adaptive pathogen, replicating and avoiding host defense mechanisms within alveolar macrophages (5). Phagocytosis of *M.tb* by alveolar macrophages is the first step in determining whether the bacillus will escape the immune response (25). *M.tb* has developed different strategies by which it enters mononuclear phagocytes and evades phagolysosome fusion. As stated above, the MR, CR3 and TLRs mediate the macrophage-mycobacterium interaction upon entry of *M.tb* within the lungs. *M.tb* phagocytosis by human macrophages via the MR-ManLAM pathway has been well documented (17,27). Entry through this route allows a safe haven for the mycobacteria to reside within altered mycobacterial phagosomes that do not mature into phagolysosomes (16). The MR is highly expressed on alveolar macrophages and the MR-mediated phagocytosis of *M.tb* is known to produce anti-inflammatory cytokines, and reduce nitric oxide and oxidant radical production (6). Thus, while the MR is efficient in removing routinely encountered airborne particles in the airways, it seems to provide a critical role for *M.tb* survival.

Human macrophages, including MDMs, also express the β-glucan receptor, Dectin-1, which has been well demonstrated to produce pro-inflammatory cytokines upon ligation with its ligand, β-glucan (34). β-glucans are present in certain fungal cell walls, and studies suggest that another ligand for Dectin-1 may exist on attenuated or avirulent

mycobacteria (13,36). However, the virulent H37Rv strain of *M.tb* does not appear to have a Dectin-1 ligand (36). Therefore, the presence of Dectin-1 ligands appears to relate to the pathogenicity of the invading *Mycobacterium spp*.

Beta-Glucan Particles as Therapeutic Agents: β-glucan is a polysaccharide commonly found in oats, barley, and yeast. Its basic structure consists of about 20-30 β-1,3-D-glucose units with branched side chains of either β-1,3 or β-1,6-glucose molecules (Figure 2). The exact structure of a β-glucan particle depends on its source, varying in solubility and branching. β-glucans are commonly found in the cell walls of fungi. Interestingly, these polysaccharides become exposed on the fungal cell wall only during the replicative periods of swelling and germ tube formation (13). Certain fungal species cause infections in humans via entry through the respiratory tract. Because of this, alveolar macrophages have adapted a mechanism for recognizing and eliminating these organisms when they are inhaled by the host and come in contact with lungs. β-glucans are recognized on lung alveolar macrophages through interaction with the non-opsonic C-type lectin, Dectin-1 as noted above (31). Among phagocytic cells, alveolar macrophages exhibit the highest surface expression of Dectin-1, speculated as an evolutionary mechanism caused by the interaction of the lung environment with fungi (30). After binding to its β-glucan ligand, Dectin-1 stimulates immune responses to combat fungal infection. β-glucan binding to Dectin-1 is known to stimulate increased TNF-α and IL-6 secretion by macrophages, resulting in phagocytosis, inflammation and antimicrobial defenses (8,13,36). Although currently a topic of debate, Dectin-1 is thought to work in coordination with TLRs to up-regulate an immune response (36). CR3

was previously identified as a β-glucan receptor, stimulating cytotoxic activity of phagocytes and host defense mechanisms (35).

Over the past thirty years β-glucan supplements have been experimentally tested to combat various human diseases. The idea is that β-glucan molecules will up-regulate an immune response which could have been suppressed or eluded by the pathogen. The most common forms of supplementary β-glucan are: insoluble whole glucan particle (WGP), a 1,3/1,6- β-glucan purified from *Saccharomyces cerevisiae* and soluble poly-1-6-β-D-glucopyranosyl-1-3- β-D-glucopyranose (PGG), formed by acid hydrolysis of WGP (19). β-glucan particles have been used as anti-tumor agents, combating metastasis through the increased production of natural killer and interferon-γ-positive cells (18). These compounds have also been found to fight against pathogens outside of the fungal realm. β-glucan has been shown to protect mice against *Streptococcus pneumoniae, Staphylococcus aureus,* and *Escherichia coli* (12,21,22). In all cases, a decreased bacterial load was observed following β-glucan treatment. In an *in vivo* study most relevant to our *in vitro* experiments, β-glucan administration has been tested on mice intravenously infected with *M. bovis* BCG. The study provided evidence that β-glucan molecules initiate a defense strategy to enhance the host immune response against mycobacterial infection (11).

M. bovis BCG is an attenuated strain of *Mycobacterium bovis,* the causative agent of TB in cattle. *M. bovis* BCG is currently used as a protective vaccine against TB in humans throughout the world, showing variable results (24). This strain retains some antigenic properties, while its virulence in humans has been attenuated (4). The genomic sequences of *M. bovis* BCG and *M.tb* are > 99.95% identical (7). The structure of the

MR ligand, ManLAM, on *M. bovis* BCG resembles that found on *M.tb*, providing evidence for similar macrophage interactions between the two strains (32). Because of their similar interactions with human macrophages, *M. bovis* BCG can be used experimentally as a model to study the effects on *M.tb* infection.

Due to the increased rates of MDR-TB and XDR-TB, new therapies are needed for TB disease. β-glucan particles PGG and WGP could bring a solution to this problem as they have shown the ability to fight various bacterial diseases including those caused by mycobacterial species. As stated above, treatment with β-glucan protected mice from *M. bovis* BCG infection (11). However, it is crucial to use human cells in order to further research and development of a therapy for human mycobacterial diseases, especially TB. Such cells are known to be significantly different from those found in rodents. In our investigation, we examined the association and antimicrobial effects that PGG and WGP have on *M. bovis* BCG upon infection of human monocyte-derived macrophages. This study provides evidence for decreased macrophage association of *M. bovis* BCG along with decreased survival within macrophages when MDMs were pretreated with the β-glucan compounds.

Methods and Materials:

Mycobacterial strains, Media and β-Glucan Compounds: *M. bovis* BCG (Pasteur strain) was seeded onto Difco™ 7H11 agar (Benton, Dickinson and Company) plates. The plates were incubated at 37ºC/5% CO_2 for 10-12 days to allow for bacterial lawn formation. These bacterial lawns were then used to prepare *M. bovis* BCG single cell

suspensions for infection of macrophages. The β-glucan compounds used in our assays were supplied by Biothera [PGG (soluble) and WGP (insoluble)] (Eaton, Minnesota).

***Preparation of Monocyte-Derived Macrophages from Human Blood (Figure 3)*:**

Monocyte-derived macrophages (MDMs) were isolated from human blood, according to the established protocol of the laboratory of Dr. Larry Schlesinger (26). Blood was drawn from healthy human donors (approved by IRB) using a syringe containing heparin (Abraxis Pharmaceutical Products, Schaumburg , IL) to prevent clotting, and then transferred to a 50 ml conical tube containing Normal Saline solution (1:1 v/v) to which was gently added Ficoll-Hypaque solution (Amersham Biosciences, Sweden) to form a layer at the bottom of the tube. The tubes were centrifuged at 500 x g for 40 minutes at room temperature to separate the "buffy coat" layer containing peripheral blood mononuclear cells (PBMCs) from the bottom layer containing red blood cells (RBCs), neutrophils and platelets and the top layer containing serum. The PBMCs were carefully removed, pooled together and washed twice in cold RPMI medium, by centrifuging first at 500 x g and then at 150 x g for 10 min each. The supernatant was aspirated and the cell pellet was resuspended in a minimal volume of RPMI (usually $1/8^{th}$ the volume of the original blood sample). The cells were counted by using a hemocytometer and adjusted with RPMI containing 20% autologous serum to a concentration of 2×10^6 PBMCs/ml. The resulting cell suspension was transferred to Teflon wells and incubated at 37ºC/5% CO_2 for 5 days to allow differentiation of PBMCs into MDMs. Autologous serum was concurrently isolated from extra blood drawn from the same donor without using heparin. Blood was allowed to clot in glass tubes at room temperature and then at

4ºC to allow separation of serum. The serum was finally harvested by centrifugation at 500 x g for 20 minutes at 4ºC.

After 5 day incubation, the PBMCs containing the differentiated population of MDMs (usually ~10% of the total PBMCs) were harvested from Teflon wells by several washes with cold RPMI and centrifugation at 150 x g for 10 min at 4ºC. The supernatants were aspirated, the pellet was resuspended in a minimal volume of RPMI and the total cells were counted by hemocytometer. The cell suspension was adjusted in RPMI containing 10% autologous serum to a concentration of 4 x 10^6 cells/ml and dispensed into a 24-well tissue culture plate (0.5 ml/well) with glass coverslips (Cell Association Assay) or without glass coverslips (Bacterial Survival Assay) in order to allow adherence of MDMs. After incubation for 2-3 h at 37ºC/5% CO_2, the adherent monolayer of MDMs (~2 x 10^5 cells/well) in each well was washed with warm RPMI in order to remove the non-adherent lymphocytes. The cells were repleted with serum-free RHH (RPMI + 10mM Hepes + 1 mg/ml human serum albumin) medium for the Cell Association Assay or with RPMI containing 20% autologous serum for an additional incubation of 7 days for the β-Glucan Toxicity and Bacterial Survival Assays.

B-Glucan Toxicity Assays: MDM monolayers in a 24-well tissue culture plate at Day 5 were repleted with 20% serum-containing RPMI and incubated for 7 more days to ensure stable adherence of cells. At Day 12, the macrophage monolayers were exposed to different concentrations of PGG or WGP (0, 6.25, 12.5, 25, 50 and 100 µg/ml in each case) in triplicate, in RPMI containing 1% serum (serum concentration was changed to 2% for Survival Assays). The morphology of the MDM monolayer was checked daily

for a period of five days under an inverted microscope and pictures taken to examine the toxicity of the β-glucan compound on macrophages. MDM viability was also assessed by using 0.4% Trypan Blue Exclusion Staining (Sigma) at the end of the 5-day period.

Preparation of Mycobacterial Single Cell Suspension for Infection: This protocol (known as "Third Tube Protocol") was established in Dr. Schlesinger's laboratory. Loopfuls of freshly-grown (10-12 days) *M. bovis* BCG from a 7H11 agar plate were re-suspended in 1.0 ml of RHH medium in an O-ring tube (Fisher Scientific) containing two ethanol-flamed glass beads. The tube was pulse-vortexed three times and allowed to sit undisturbed at room temperature for 30 min. The upper portion of the suspension (~700 µl) was removed, transferred to a second O-ring tube and allowed to settle for 5 min. The upper portion of the suspension (~600 µl) was again transferred to a third tube. Dilutions were made of the final suspension and bacterial cells were counted by using a Petroff-Hausser counting chamber (Hausser Scientific, Horsham, PA). The bacterial suspension was adjusted in RHH to a concentration of 4×10^7 bacteria/ml for the Cell Association Assay and 4×10^6 bacteria/ml for the Bacterial Survival Assay.

Cell Association Assay: Monolayers of 5 day-old MDMs on coverslips in a 24-well tissue culture plate were washed with warm RPMI, repleted with 450 µl RHH containing 50 µg/ml of β-glucan PGG or WGP and incubated for 30 min at 37ºC/5% CO_2. Then, 50 µl of an *M. bovis* BCG single cell suspension (4×10^7 bacteria/ml) was added to each well (Multiplicity of Infection or MOI = 10:1 bacteria per cell) and incubated with shaking for 30 min at 37ºC/5% CO_2, followed by an additional incubation without shaking for 90

minutes at 37ºC/5% CO_2 (total 2 h). The infected monolayer was washed with warm RPMI and fixed with 10% formalin (Sigma, St. Louis, MO) in PBS buffer for 10 min. The monolayer was then washed with PBS and kept at 4ºC until Auramine-Rhodamine Staining. Fixed MDM monolayers on coverslips were flooded with Auramine-Rhodamine stain (Becton, Dickinson and Company, Sparks, MD) and incubated at room temperature in the dark for 20 min. The coverslips were dipped in H_2O to stop staining and covered with acid-alcohol (700 ml 100% ethanol + 5 g NaCl + 300 ml deonized H_2O + 5 ml conc. HCl) for 3 min in the dark. The coverslips were rinsed again in H_2O, flooded with 0.5% $KMnO_4$ for 2 min and finally mounted with 50% glycerol on slides. The non-β-glucan control, PGG- and WGP-pretreated MDM-containing coverslips were used in triplicate for each test group. In each group, the MDM-associated *M. bovis* BCG bacterial cells were counted (≥ 300 MDMs) under a fluorescent microscope (Olympus BX51) and reported as average bacteria/macrophage. One tailed t-tests were performed to determine the significance of the results between groups.

Mycobacterial Survival Assay: Monolayers of 12 day-old MDMs in a 24-well tissue culture plate were washed with warm RPMI, repleted with 450 µl RHH containing 50 µg/ml of β-glucan PGG or WGP and incubated for 30 min at 37ºC/5% CO_2. Then, 50 µl of an *M. bovis* BCG single cell suspension (4 x 10^6 bacteria/ml) was added to each well (MOI = 1:1 bacteria per cell) and incubated with shaking for 30 min at 37ºC/5% CO_2, followed by an additional incubation without shaking for 90 minutes at 37ºC/5% CO_2 (total 2 h). The infected monolayer was washed with warm RPMI, repleted in RPMI containing 2.0% autologous serum and incubated at 37ºC/5% CO_2 for different time periods (24 h, 48 h, 72 h). At the end of each time period, the supernatant from each well

was pipetted into an O-ring tube and centrifuged at 4ºC (10,000 x g) for 10 min. The pellet was resuspended in 560 µl 7H9 broth at room temperature. Ice-cold sterile dH_2O (300 µl) containing 500 µg/ml of DNase was added to each well and let stand for 10 min. The resuspended pellet in 7H9 was added to its corresponding well along with 240 µl of 0.25% SDS solution in PBS. The plate was periodically shaken for 10 min in order to ensure complete lysis of the MDM monolayers. Then, 300 µl of 20% Bovine Serum Albumin (BSA) in PBS was added to each well for neutralization. The contents of each well were placed into an O-ring tube with two flame-sterilized glass beads and pulse-vortexed five times. Appropriate dilutions (usually, 20, 40, 80, 160) of the lysates were made in 7H9 containing OADC (Oleic Acid-Dextrose-Catalase) and plated on 7H11 agar quadrant plates. The plates were incubated at 37ºC/5% CO_2 until bacterial colonies appeared (18- 21 days) which were counted as colony forming units (CFUs). The non-β-glucan control, PGG- and WGP-pretreated MDM test groups were performed in triplicate. CFUs were determined for each group and averages were used to perform one tailed t-tests to assess the significance of the results between test groups.

Results:

Beta-Glucan Toxicity of Human Macrophages: The toxicity of PGG and WGP on the MDM monolayer was tested for a period of 5 days using different β-glucan concentrations (6.25, 12.5, 25, 50, and 100 µg/ml for each) in the medium. MDM monolayers without the addition of a β-glucan preparation were used as the control. Cell morphology and the integrity of the monolayer were checked daily under an inverted

microscope and cell death was finally examined by Trypan Blue Exclusion staining at the end of the 5-day period. Results showed that both PGG and WGP at any concentration had no toxic effect on MDMs at day 1 and 2. Figure 4 shows no difference in cell morphology between the control groups and the PGG- and WGP-pretreated groups. However, at day 3, the MDM monolayer treated with 100 μg/ml of WGP showed partial lifting and progressively more cell death through Day 5. At this time point, WGP (100 μg/ml) proved to be either completely or partially toxic to the monolayers, while all other monolayers appeared healthy throughout the five-day period (Figure 5). Trypan Blue Exclusion staining of cell monolayers at day 5 showed a similar pattern of results as observed by microscopy (Table 1). At day 5, about 80% cell death was caused by the use of 100 μg/ml WGP, while only 9% cell death occurred at 50 μg/ml. All other WGP concentrations exhibited no cellular death. PGG at 100 μg/ml showed 6% cell death only at day 5, while all other concentrations of PGG showed no effect on the MDM monolayer. These results were used to choose an appropriate concentration of β-glucan preparation for bacterial cell association and survival assays.

M. bovis BCG Association with β-Glucan-Treated Human Macrophages: Cell association experiments were performed by using MDMs from two different donors (n = 2) with and without β-glucan in triplicate wells for each test group. β-glucan (PGG or WGP) was used at 50 μg/ml concentration. Both experiments showed similar trends with respect to decreased *M. bovis* BCG association with macrophages pre-treated with β-glucan preparations (Figures 6A and 7A). In Experiment 1, the association results for β-glucan-treated MDMs were examined by determining the percent association when compared to the non-glucan control. In Figure 6B, the percent of control comparison

shows a significant ($P < 0.0001$) decrease (35%) in bacterial association with PGG-treated MDMs. Similarly, WGP-treated MDMs show a significant ($P < 0.005$) decrease (43%) in *M. bovis* BCG association compared to the non-β-glucan control. Experiment 2, using a different donor, produced similar results demonstrating the reproducibility of the assay. The percent of control results (Figure 7B) for both PGG- and WGP-treated MDMs show decreased bacterial association (33% for both) with macrophages and are statistically significant (PGG, $P = 0.0005$; WGP, $P < 0.005$). These results show that β-glucan limits bacterial binding to and uptake into the macrophage, perhaps through pre-engagement of the macrophage receptor CR3 by β-glucan particles.

M. bovis BCG Survival in β-Glucan-Treated Human Macrophages: A mycobacterial survival assay was performed by infecting β-glucan-treated MDMs wth *M. bovis* BCG and allowing bacterial replication within macrophages over a 3-day period. Each day post-infection, the infected MDMs were lysed, plated on agar medium, colonies counted after 21 days and expressed in CFUs/ml (Figure 8). At 24 h and 48 h post-infection, there was no significant difference in the number of CFUs of *M. bovis* BCG between the β-glucan-treated and non-treated groups (Figure 8A and 8B). However, 72 h post-infection, a significant decrease in CFUs was observed in the β-glucan-treated groups compared to the control group (Figure 8C). Figure 8D shows a growth curve of *M. bovis* BCG over the 3-day period. The CFU results at 72 h were then examined by determining the percent CFUs of both β-glucan groups compared to the non-glucan control (Figure 8E). The percent control results for PGG and WGP CFUs show a significant decrease in *M. bovis* BCG survival in macrophages by 54% ($P < 0.005$) and 74% ($P < 0.001$),

respectively. These results show an anti-mycobacterial activity conferred by human macrophages when treated with β-glucans.

Discussion:

M.tb is an intracellular pathogen which is highly adapted to evade host defense mechanisms and replicate within alveolar macrophages. The gradual increase in MDR-TB and the recent emergence of XDR-TB throughout the world add to the difficulty in controlling TB disease. Patients with MDR-TB have a fatality rate of 40-60%, the same rate of death for non-treated TB cases (14). The increasing rate of drug-resistant forms of TB highlights the need for new therapies to combat the disease. Recently 1,3/1,6-β-D-glucan particles have been identified as an agent in cancer treatment. Studies have shown that β-glucan binds to CR3 on leukocytes in a non-opsonic manner and stimulates cytotoxic activation of C3bi-opsonized tumor cells by CR3 (35). Without β-glucan binding, CR3 is unable to mediate lysis of C3bi-opsonized tumor cells (33). These findings show one mechanism by which β-glucan particles promote anti-tumor activities.

Yeast-derived 1,3/1,6-β-D-glucans have also been shown to stimulate immune responses and microbicidal activities. Mice pre-treated with β-glucan showed an increased leukocyte count along with increased production of TNF-α and IL-6 pro-inflammatory cytokines and respiratory burst (9,20). In recent studies, β-glucans showed a protective effect against *S. pneumoniae* when mice were treated with β-glucan prior to or after infection with the bacteria (12). Similarly, PGG and WGP have been shown to have a protective effect against anthrax infection, caused by the bacterium *Bacillus*

anthracis (19). Intravenously injected β-glucan particles significantly reduced the bacterial load within the lungs of mice, while also increasing the survival rate of the animal.

Our findings show a similar protective effect of PGG and WGP on human macrophages from *M. bovis* BCG infection. Decreased cellular association and bacterial survival within MDMs were seen in the presence of β-glucan particles (Figures 6 through 8). The survival assay shows that MDM microbicidal activity against *M. bovis* BCG is enhanced in the presence of PGG and WGP (Figure 8). Less bacterial survival within macrophages reduces the pathogen's ability to replicate and cause infection in a host. The decreased effect that β-glucan has on cellular association also limits the pathogenicity of *M. bovis* BCG. Without binding to and entry into the macrophage, *M. bovis* BCG and presumably *M.tb* strains have less ability to evade host defense mechanisms, since intracellular replication is a key to effective infection.

Although no mechanisms of killing were investigated in our study, prior investigations give us insight to speculate about the function that the β-glucans have on human macrophages when infected with mycobacterial strains. Increased TNF-α production is known to stimulate inflammatory responses along with increased nitric oxide and respiratory burst activities (25). Fungal pathogens are recognized by alveolar macrophages through interaction with the β-glucan receptor Dectin-1, resulting in increased inflammatory responses (31). Dectin-1 is thought to work in co-operation with TLR-2 to stimulate TNF-α production (36). The pre-incubation of MDMs with β-glucan allows for binding of this carbohydrate to Dectin-1 on the macrophage surface, resulting in the increased production of pro-inflammatory responses which could be the major

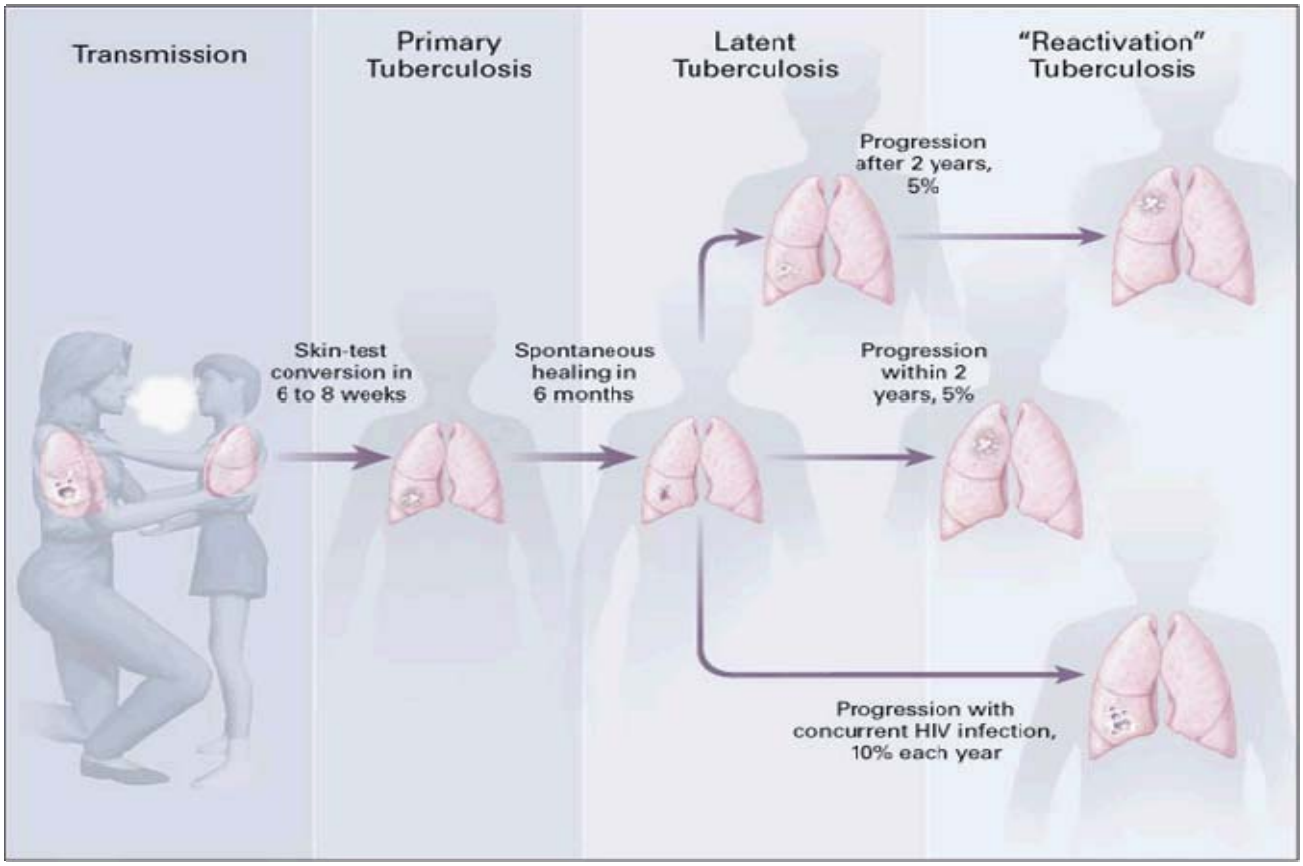
cause of bactericidal killing of *M. bovis* BCG in our study (Figure 8). As stated above, β-glucan also primes CR3 on leukocytes for cytotoxic killing of C3bi-opsonized particles in mice (35). Although not much is known about *M.tb*'s pathogenic dependency on CR3, it has been reported that the bacterium is phagocytosed by macrophages in a C3bi-opsonic manner (5). It is possible that β-glucan particles bind to and activate CR3 to lyse *M. bovis* BCG, resulting in decreased survival of the bacteria within MDMs. The pre-occupation of CR3 (especially, its lectin-binding site) on MDMs by β-glucan is likely responsible for reduced association of *M. bovis* BCG with MDMs as observed in our study (Figures 6 and 7).

When compared to monocytes from patients with active TB, monocytes from MDR-TB patients show a decrease in TNF-α and NO production after infection with *M.tb* (28). It is now generally agreed that *M.tb* infection dampens the host immune response and allows the bacterium to replicate inside the host cell. TNF-α has been demonstrated as a key component in eliciting the protective host immune response to TB infection (25). Since β-glucan is known to stimulate TNF-α production through binding to Dectin-1, this complex carbohydrate could initiate an immune response to defend against MDR-TB. The data suggest the possibility that β-glucans may act as an agent to control the increasing rate of drug-resistant TB strains, where boosting of host immunity is the only alternative to the use of antibiotics.

To our knowledge, this study was the first to test the effects of β-glucan on mycobacterial survival in human macrophages, and the slow-growing *M. bovis* BCG was used here as a model for the TB bacillus. Because of the physiological and genomic similarities between *M. bovis* BCG and *M.tb*, these results indicate promising effects for

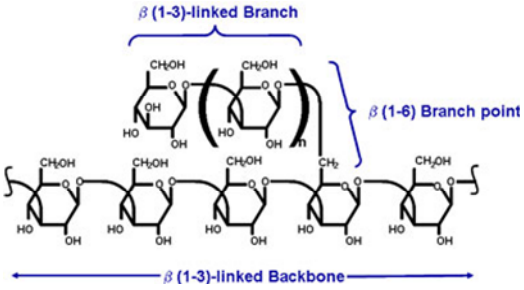
the potential protective role of β-glucans for *M.tb* infection of human macrophages. The use of human macrophages is crucial in testing and validation of β-glucans as a possible therapeutic agent. One of the future directions of our project will be to study the effects of PGG and WGP on human alveolar macrophages infected with virulent *M.tb* strains. Treatment of macrophages with β-glucan at different time points after infection with *M.tb* will also be done in the future study as the post-infection period is the time when drugs are administered to patients. Though further investigations are needed, our preliminary results provide evidence for potential use of β-glucans as an alternative therapeutic agent to control the spread of TB.

Figures:

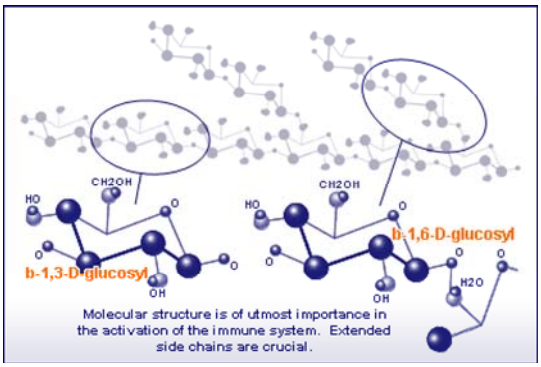


(Small and Fujiwara, 2001)

Figure 1- Tuberculosis (TB) disease pathogenesis. The first step in TB disease is transmission of the causative bacterium, *Mycobacterium tuberculosis (M.tb)*. Transmission occurs when aerosol droplets containing *M.tb* from an individual with active TB in the lungs are deposited into the lungs of a healthy host. Primary TB infection causes lesions in the lungs and elsewhere called granulomas by recruiting activated immune cells. The host's immune system is able to suppress replication of the bacterium and cause healing, leading to latent TB. Latent TB can then lead to "reactivation" TB if the individual's immune system is compromised, resulting in uncontrolled replication of *M.tb* (29).



Picture obtained from http://www.immunealert.com/



Obtained from http://immunocorp.com/images/molly.gif

Figure 2- β-glucan structures. β-glucans are polysaccharides with a main chain 1,3-D-glucopyranose and 1,6-D-glucopyranose branches. Poly-1-6-β-D-glucopyranosyl-1-3-β-D-glucopyranose (PGG) is a soluble form of β-glucan commonly used as a therapy for selected human diseases. Whole glucan particle (WGP) is the insoluble form of PGG.

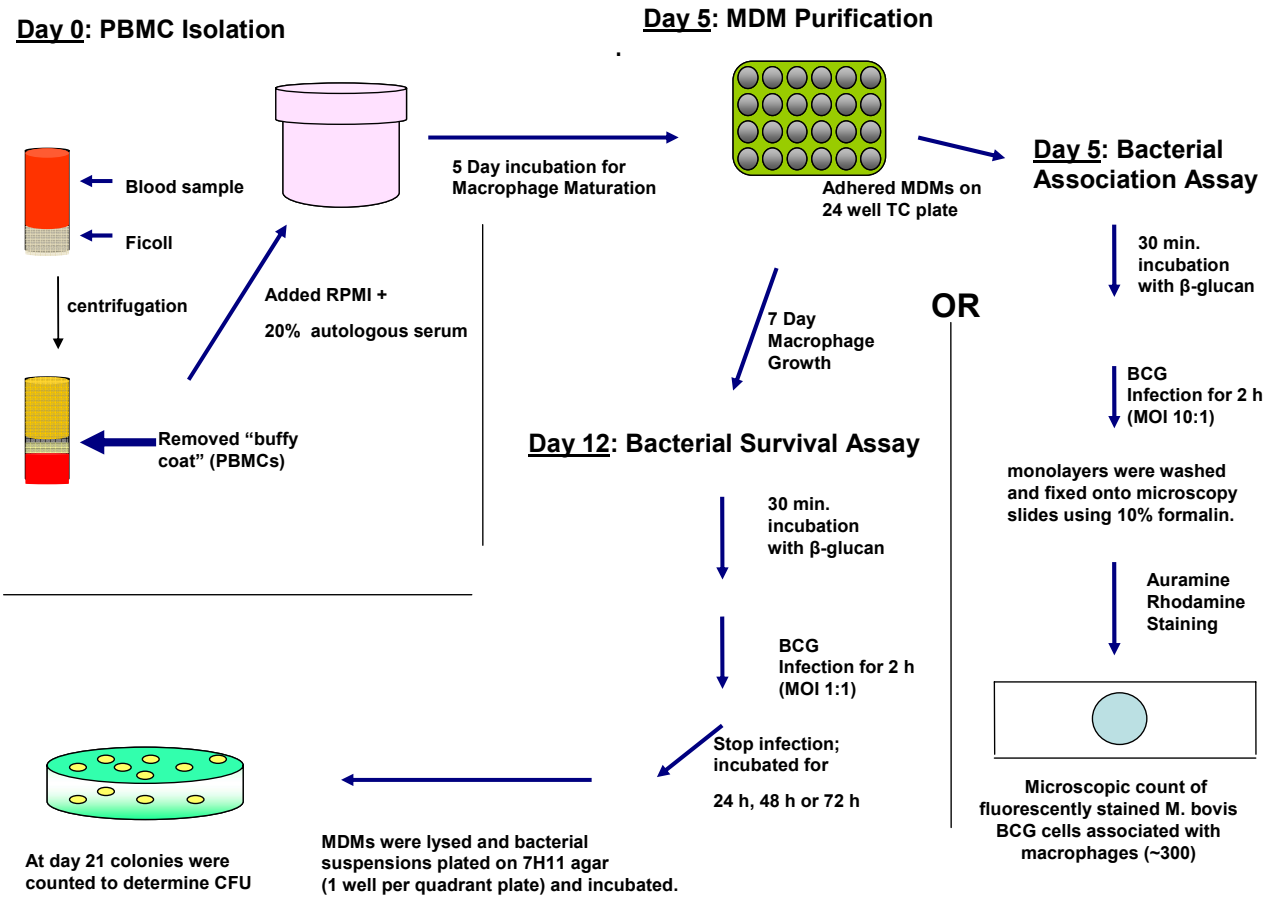


Figure 3- Methods of isolation and infection of monocyte-derived macrophages (MDMs) with *M. bovis* BCG. Peripheral Blood Mononuclear Cells (PBMCs) were isolated on Day 0 from human donors and incubated for 5 days to allow differentiation into MDMs. At day 5, MDM monolayers were adhered to coverslips (Bacterial Association Assay), or incubated for another 7 days for performing the Bacterial Survival Assay. Each assay involved 30 min pre-incubation of MDMs with either PGG or WGP and 2 h incubation with *M. bovis* BCG.

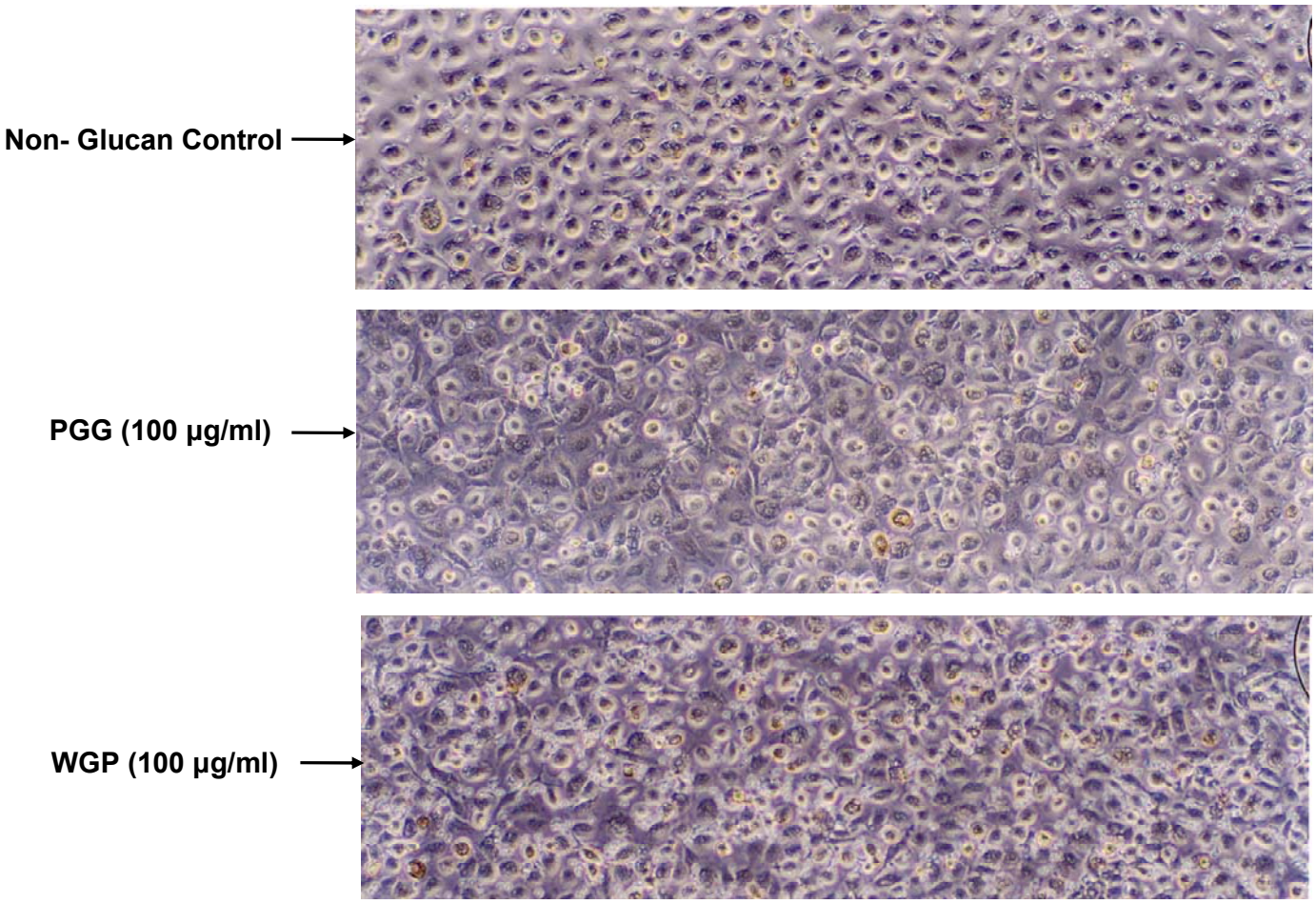
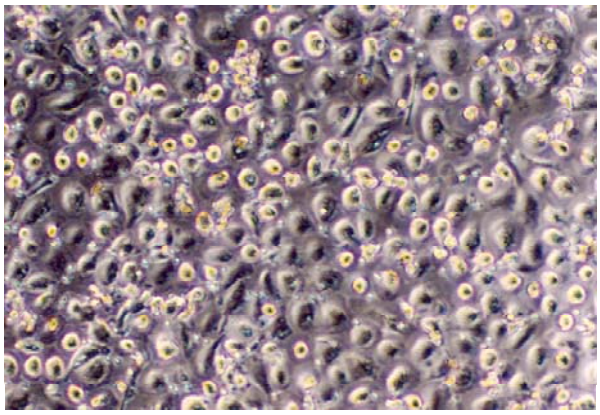
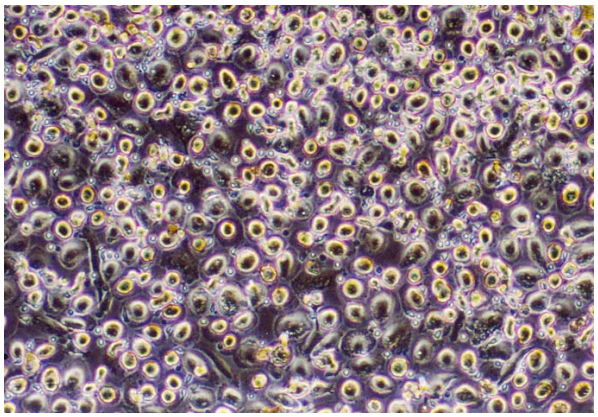


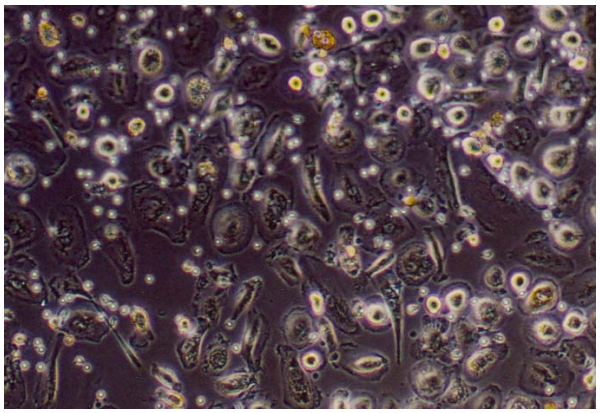
Figure 4 – Human macrophage monolayers at day 1 of exposure to β-glucan preparations. Day-1 MDM monolayers appeared healthy and intact for all concentrations of PGG and WGP. There was no noticeable change in morphology of MDMs between the non-glucan- and β-glucan-treated groups.



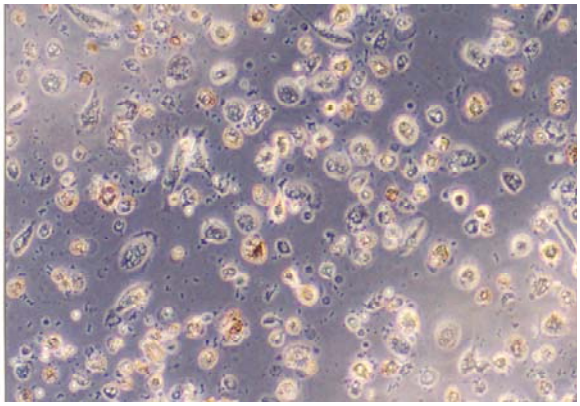
Non- Glucan Control



PGG (100 µg/ml)



WGP (100 µg/ml): partially toxic



WGP (100 µg/ml): completely toxic

Figure 5- Human macrophage monolayers at day 5 of exposure to β-glucan preparations. At Day 5, WGP (100 µg/ml) proved partially or completely toxic for the MDMs. All other concentrations of WGP had no significant effect on the monolayer. However, MDM pre-treatment with all concentrations of PGG exhibited no significant effect on the monolayer compared to the non-glucan control.

Table 1

β-Glucan	Concentration (µg/ml)	Mean Cell Death* (%)
PGG	0.0	0
	6.25	0
	12.5	0
	25.0	0
	50.0	0
	100.0	6
WGP	0.0	0
	6.25	0
	12.5	0
	25.0	0
	50.0	9
	100.0	80

*Mean of duplicate wells (200 cells)

0.4% Trypan Blue Staining was used to assess cellular death in β-glucan pre-treated MDM monolayers at day 5. WGP (100 µg/ml) showed high toxicity for human macrophages with nearly 80% cell death at Day 5. All other concentrations of PGG and WGP had little or no effect on macrophage death.

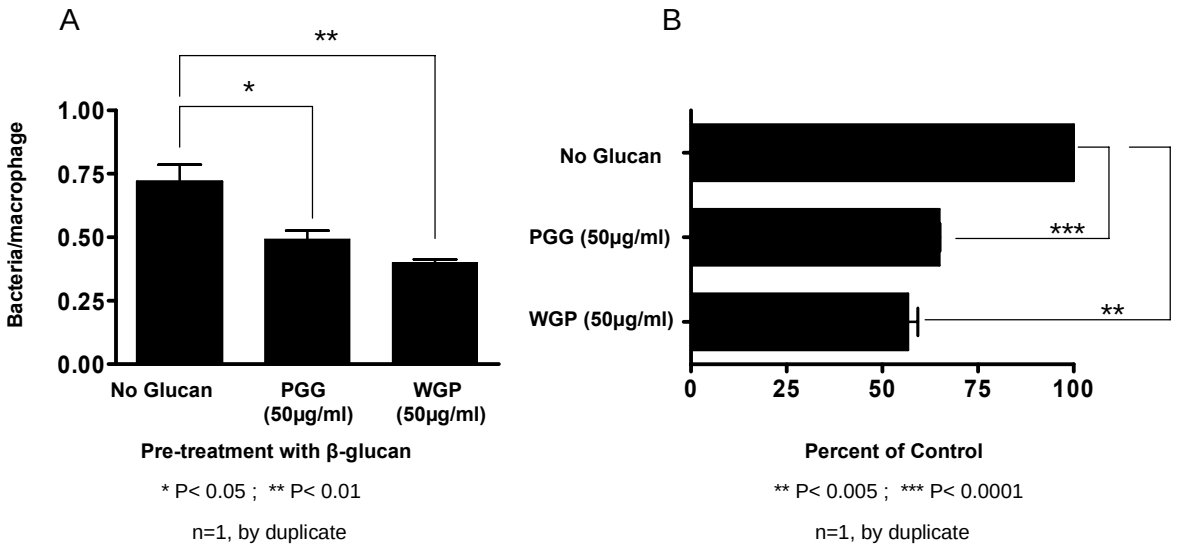


Figure 6- *M. bovis* BCG association with β-glucan treated MDMs (Experiment 1). MDMs in each well (2 x 10^5 cells) of a 24-well tissue culture plate were incubated at 37ºC/5% CO_2 in the absence or presence of PGG or WGP (50 µg/ml). MDMs were then infected with 2 x 10^6 *M. bovis* BCG (MOI = 10:1) at 37ºC/5% CO_2 for 2 h. The infection was stopped and the cells were fixed. Auramine Rhodamine staining was performed and cell-associated bacteria were counted under a fluorescence microscope. A) Mean Bacteria/Cell Association: β-glucan stimulation caused significantly less cellular association of *M. bovis* BCG with human macrophages compared to the non-glucan control; B) Percent of Control Comparison: Compared to the non-glucan control, PGG and WGP significantly reduced bacterial association with MDMs.

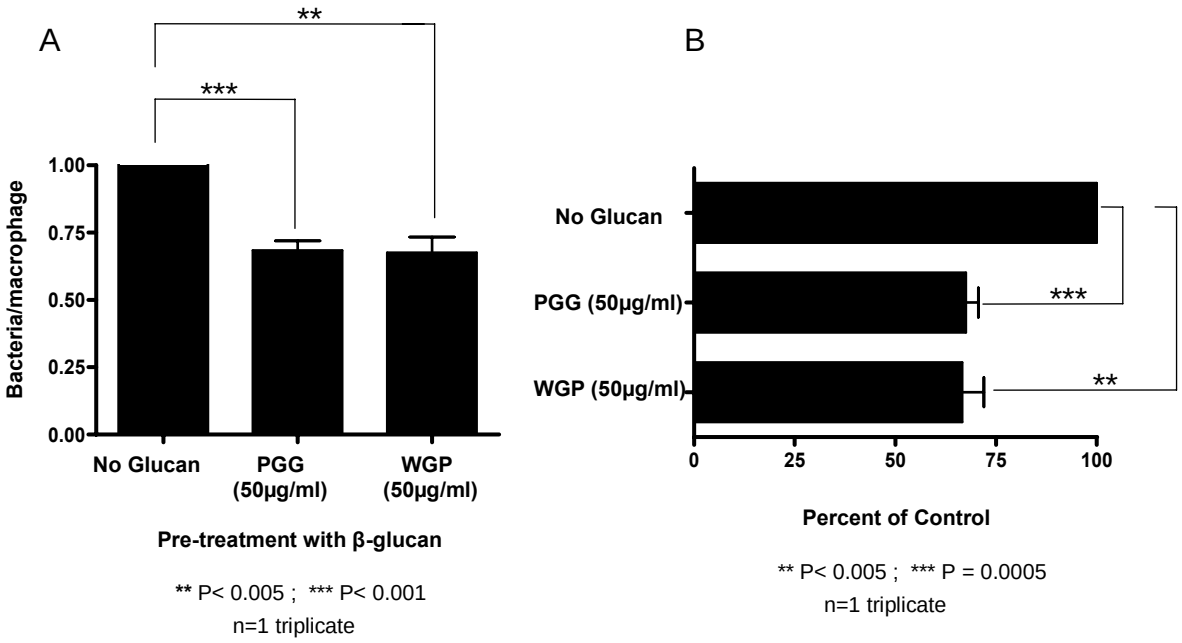


Figure 7- *M. bovis* BCG association with β-glucan treated MDMs (Experiment 2). MDMs in each well (2 x 10^5 cells) of a 24-well tissue culture plate were incubated at 37ºC/5% CO_2 in the absence or presence of PGG or WGP (50 µg/ml). MDMs were then infected with 2 x 10^6 *M. bovis* BCG (MOI = 10:1) at 37ºC/5% CO_2 for 2 h. The infection was stopped and the cells were fixed. Auramine Rhodamine staining was performed and cell-associated bacteria were counted under a fluorescence microscope. A) Mean Bacteria/Cell Association: β-glucan stimulation caused significantly decreased cellular association of *M. bovis* BCG with human macrophages compared to the non-glucan control; B) Percent of Control Comparison: Compared to the non-glucan control, both PGG and WGP significantly reduced the association of *M. bovis* BCG with MDMs.

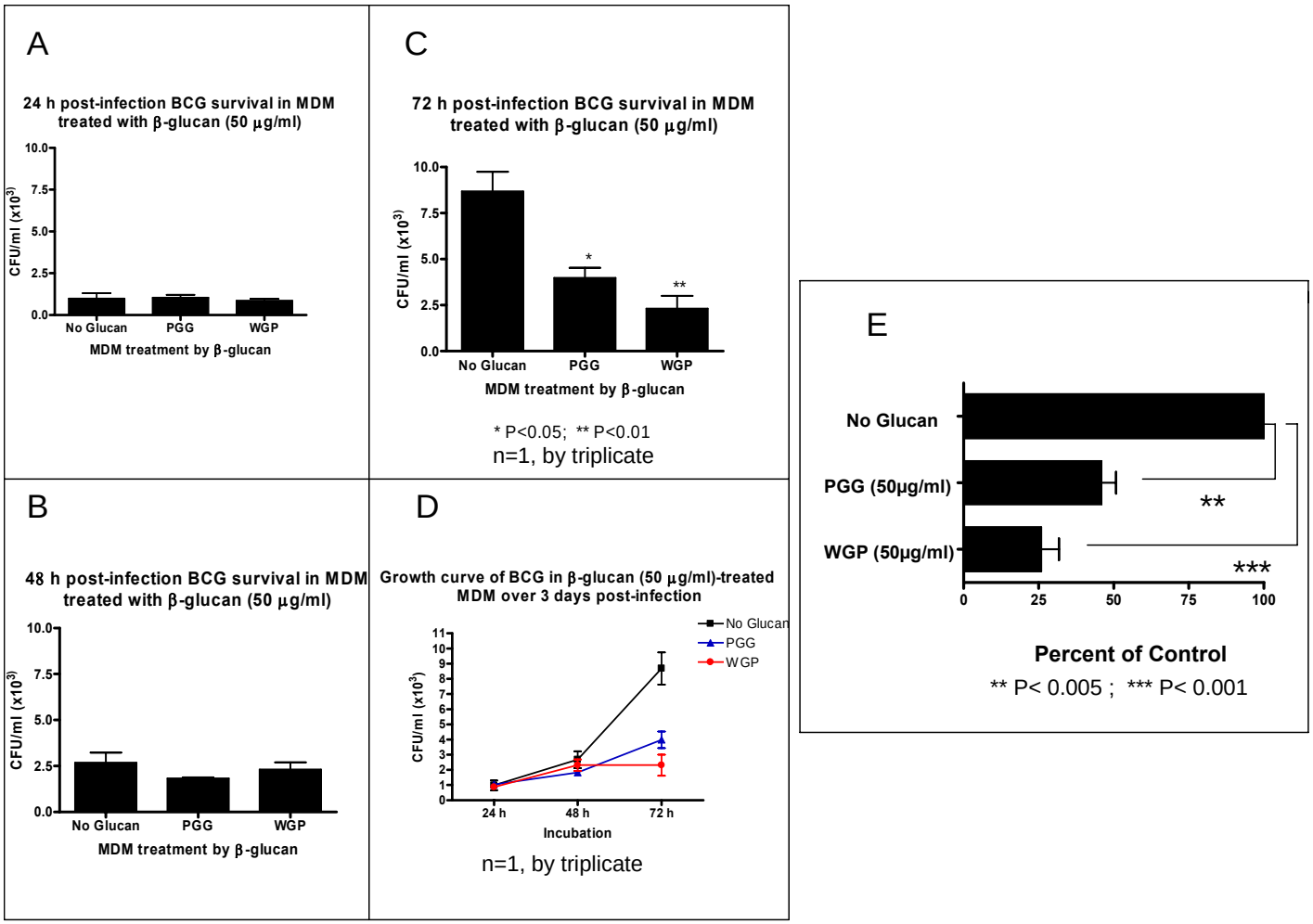


Figure 8- *M. bovis* BCG survival within β-glucan treated MDMs. MDMs in each well (2 x 10^5 cells) of a 24-well tissue culture plate were incubated at 37ºC/5% CO_2 in the absence or presence of PGG or WGP (50 µg/ml). MDMs were then infected with 2 x 10^5 *M. bovis* BCG (MOI = 1:1) and incubated at 37ºC/5% CO_2 for 2 h. The infection was stopped with washing of the non-adherent bacteria and the cells were incubated in RPMI + 2.0 % autologous serum for 24 h, 48 h or 72 h. At each time point, cells were lysed and lysates plated on 7H11 agar in different dilutions and incubated at 37ºC/5% CO_2 for growth. Bacterial colonies were counted at 21 days and expressed in CFU/ml. A and B) CFUs of *M. bovis* BCG were not affected by PGG or WGP 24 h or 48 h post-infection periods; C) At 72 h, CFUs were significantly decreased in the presence of PGG and WGP, indicating decreased intracellular survival of *M. bovis* BCG in macrophages treated with β-glucan. D) The growth curve of *M. bovis* BCG shows inhibition of growth at 72 h in PGG and WGP pre-treated macrophages compared to the non-glucan control. E) Percent of Control Comparison: Compared to the non-glucan control, both PGG and WGP treatment showed a significant decrease in bacterial growth in MDMs.

Acknowledgements:

I would like to specially thank Dr. Abul Azad and Dr. Larry Schlesinger for their time and mentoring throughout this project. I would also like to thank Dr. Jordi Torrelles, for his training of many of the techniques used, along with the rest of the Schlesinger Laboratory, for all of their help over the past nine months.

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